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Phylogenetic Utility of the Internal Transcribed Spacers of Nuclear Ribosomal DNA in Plants: An Example from the Compositae

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The internal transcribed spacer (ITS) region of 18–26S nuclear ribosomal DNA was sequenced in 12 representatives of the Compositae subtribe Madiinae and two outgroup species to assess its utility for phylogeny reconstruction. High sequence alignability and minimal length variation among ITS 1, 5.8S, and ITS 2 sequences facilitated determination of positional homology of nucleotide sites. In pairwise comparisons among Madiinae DNAs, sequence divergence at unambiguously aligned sites ranged from 0.4 to 19.2% of nucleotides in ITS 1 and from 0 to 12.9% of nucleotides in ITS 2. Phylogenetic relationships among ITS sequences of Hawaiian silversword alliance species (*Argyroxiphium, Dubautia,* and *Wilkesia*) and California tarweed taxa in *Adenothamnus, Madia, Raillardella,* and *Raillardiopsis* are highly concordant with a chloroplast DNA-based phylogeny of this group. Maximally parsimonious trees from ITS and chloroplast DNA data all suggest (a) origin of the monophyletic Hawaiian silversword alliance from a California tarweed ancestor, (b) closer relationship of the Hawaiian species to *Madia* and *Raillardiopsis* than to *Adenothamnus* or *Raillardella,* (c) paraphyly of *Raillardiopsis,* a segregate of *Raillardella,* and (d) closer relationship of *Raillardiopsis* to *Madia* and the silversword alliance than to *Raillardella.* These findings indicate that the ITS region in plants should be further explored as a promising source of nuclear phylogenetic markers. © 1992 Academic Press, Inc.

Chloroplast DNA (cpDNA) provides an important source of characters for phylogeny reconstruction in plants (cf. Palmer *et al.,* 1988; Clegg and Zurawski, 1991). Mounting evidence demonstrates, however, that species-lineage reconstructions using this cytoplasmically inherited DNA molecule may be prone to significant error from hybridization and introgression events or lineage sorting (reviewed in Rieseberg and Soltis, 1991; Doyle, 1992). Fortunately, comparison of cpDNA and nuclear DNA phylogenies offers a means of identifying such problems (Smith and Sytsma, 1990; Wendel *et al.,* 1991; Rieseberg, 1991) and verifying species relationships (Sytsma and Schaal, 1985; Rieseberg *et al.,* 1988; Wallace and Jansen, 1990). In addition, such comparisons can significantly improve understanding of the origin of polyploid species (Soltis and Soltis, 1991).

Despite the enormity of the nuclear genome, identification of nuclear DNA regions that are useful for phylogenetic comparisons with cpDNA data has been problematic. Such nuclear DNA regions must be (a) evolutionarily conservative—evolving primarily by point mutations at a level comparable to that in cpDNA; (b) phylogenetically interpretable—evolving in a manner that allows resolution of speciation events; (c) easily examined in the laboratory; and (d) sufficiently large to offer enough potentially useful characters for phylogeny reconstruction.

The second criterion is especially difficult to meet. Sexual recombination and segregation in nuclear DNA complicate assessment of the phylogenetic utility of molecular variation at a locus. Molecular character states that differentiate two species could represent mutations that have arisen since divergence from a common ancestor. Such characters are relevant to phylogeny. Alternatively, these different character states could predate the species split, i.e., were present in the ancestral species but were differentially transmitted to, or lost in, the daughter species. Characters of this type can be phylogenetically misleading (Roth, 1991; Pamilo and Nei, 1988; Takahata, 1989; Wu, 1991). Additionally, the presence of multiple copies of a gene at different loci or as a multigene family presents problems of homology assessment, i.e., in determining whether the sequences being compared are paralogous or orthologous (Fitch, 1970).

For assessing phylogeny in plants, 18–26S nuclear ribosomal DNA (nrDNA) has been used extensively at both the family and higher levels by DNA sequencing (cf. Hamby and Zimmer, 1991; Nickrent and Franchina, 1990) and among closely related genera or species by restriction site analysis (e.g., Sytsma and Schaal, 1985; Rieseberg *et al.,* 1988; Smith and Syt-

Sequence data from this article have been deposited with the GenBank Data Libraries under Accession Nos. M93787—M93800.

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sma, 1990; Crisci *et al.*, 1990; Kim and Mabry, 1991). The tandem repeat structure and extremely high copy number of nrDNA (cf. Rogers and Bendich, 1987) make it especially easy to detect or clone in the laboratory. Most importantly, considerable research indicates that this gene family undergoes rapid concerted evolution (cf. Zimmer *et al.*, 1980; Arnheim *et al.*, 1980; Arnheim, 1983), within and even between loci (e.g., Arnheim *et al.*, 1980; Arnheim, 1983; Appels and Dvorak, 1982; Hillis *et al.*, 1991), promoting its usefulness for phylogeny reconstruction (Sanderson and Doyle, 1992).

Variation in 18–26S nrDNA among closely related plant species, i.e., the level of most cpDNA restriction site studies, has been detected largely within the intergenic spacer (IGS) and internal transcribed spacer (ITS) regions (cf. Appels and Dvorak, 1982; Sytsma and Schaal, 1985; Kim and Mabry, 1991). The plant IGS is sufficiently large to allow sequence analysis using restriction enzymes. Much of this spacer, however, has a subrepeat structure, making it especially susceptible to length mutations (reviewed in Jorgansen and Cluster, 1988). The IGS can thus vary extensively in length among species, at the population level, or even within single individuals (reviewed in Rogers and Bendich, 1987; Schaal and Learn, 1988; see also Kim and Mabry, 1991; Zimmer *et al.*, 1988; Molnar *et al.*, 1989; Bellarosa *et al.*, 1990; D'Ovidio, 1990; Reddy *et al.*, 1990). In contrast, the ITS region has been shown to be evolutionarily conservative in length but is so small in plants that few restriction sites generally occur within it. Sequencing of the ITS region, however, has exciting potential as a source of nuclear DNA characters for phylogenetic reconstruction in plants. This promise was heightened recently by encouraging results from ITS sequence-based phylogenies of protoctistans (Lee and Taylor, 1991) and apes and humans (Gonzalez *et al.*, 1990b).

White *et al.* (1990) have taken advantage of polymerase chain reaction (PCR) technology to promote sequencing of nrDNA in fungi. This has included development of primers to allow sequencing of the entire ITS region. In this paper, I describe (a) the usefulness of these primers for PCR amplification and sequencing of the ITS region in angiosperms and (b) the utility of ITS DNA sequences as a source of phylogenetic data in the subtribe Madiinae of Compositae.

MATERIALS AND METHODS

Plant Samples

Total DNAs representing 14 populations in *Adenothamnus, Argyroxiphium, Arnica, Dubautia, Hulsea, Madia, Raillardella, Raillardiopsis,* and *Wilkesia* were isolated from fresh leaves of one or more individuals (Table 1) and purified on cesium chloride gradients, following a modification of Palmer (1986) that omitted separation of organelles.

TABLE 1

Collections Examined for 18–26S Nuclear Ribosomal DNA Internal Transcribed Spacer Nucleotide Sequence Variation

Adenothamnus validus (Brandegee) Keck—*M. S. Witter 86-99* (I), Punta Banda, S of Ensenada, Baja California, Mexico.

Argyroxiphium caliginis C. Forbes—*BGB 660* (I), Pu'u Kukui, West Maui Mountains, Maui, Hawaii.

**Arnica mollis* Hook.—*BGB 680* (P), 0.25 mile SW of Winnemucca Lake, Alpine County, California.

Dubautia arborea (A. Gray) Keck—*BGB 527* (P), Pu'u La'au, Mauna Kea, Hawaii.

Dubautia menziesii (A. Gray) Keck—*BGB 522* (P), 0.2 miles S of Haleakala National Park Headquarters along Haleakala Highway, East Maui, Hawaii.

**Hulsea algida* A. Gray—*BGB 678* (P), W slope of Round Top, near summit, Alpine County, California.

Madia bolanderi (A. Gray) A. Gray—*BGB 506* (P), Yuba Pass along State Highway 49, Sierra County, California; *BGB 509* (P), ca. 0.1 mile north of D. L. Bliss State Park along State Highway 89, El Dorado County, California.

Madia elegans D. Don subsp. *densifolia* (E. Greene) Keck—*BGB 547* (P), 3.3 miles S of junction with State Highway 92 along Canada Road, San Mateo County, California.

Madia stebbinsii Nelson and Nelson—*BGB 611* (P), 4.7 miles E of junction with Wildwood–Mad River Road along U.S. Forest Service Road 28N10, Trinity County, California.

Raillardella pringlei E. Greene—*BGB 608* (P), along outlet creek of Toad Lake, Mount Eddy region, Siskiyou County, California.

Raillardiopsis muirii (A. Gray) Rydb.—*BGB 618* (P), W slope of Ventana Double Cone, near summit, Monterey County, California.

Raillardiopsis scabrida (Eastw.) Rydb.—*BGB 676* (P), ridge east of Hull Mountain summit, Lake County, California.

Wilkesia gymnoxiphium A. Gray—*Char 76.022* (I), Iliau Loop Trail, Waimea Canyon rim, Kauai, Hawaii.

Note. DNA samples from each population were from 1 individual (I) or from 2 to 10 pooled individuals (P). Outgroup species are indicated by an asterisk. Collector abbreviation: *BGB*, B. G. Baldwin *et al.* Vouchers are at DAV (*BGB* and *M. S. Witter* collections) or HAW (*Char* collection).

Sequencing Strategy

Single-stranded DNAs of the complete ITS region in each genomic DNA (5′26S–3′18S) were directly amplified by 40 cycles of asymmetric PCR using the primers "ITS5" and "ITS4" (White, 1990; Fig. 1) in a 1:20 ratio. Single-stranded DNAs that included all of the ITS 2 region and part of the 5.8S and 26S sequences (5′26S–3′5.8S) were similarly generated by use of the primers "ITS3" and "ITS4" (White, 1990; Fig. 1). With several genomic DNAs, the primers "ITS5" and "ITS2" (White, 1990; Fig. 1) were used as above to amplify single-stranded DNAs of the ITS 1 region (5′5.8S–3′18S) for resequencing.

These 25-μl reactions contained (in order of addition) 4.28 μl of sterile water, 1.25 μl of glycerin, 2.5 μl of 10× *Taq* polymerase reaction buffer, 2.5 μl of 200 μM dNTPs in an equimolar ratio, 0.1 μl of *Taq* DNA poly-

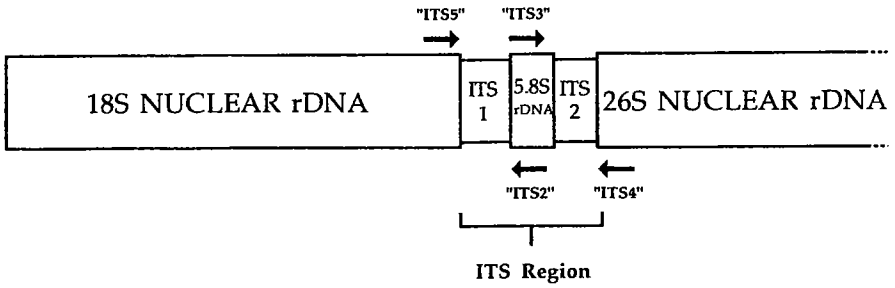

FIG. 1. Repeat unit of 18–26S nuclear ribosomal DNA, minus the intergenic spacer and much of the 26S subunit. ITS Region = internal transcribed spacer region. Arrows indicate approximate positions of primers used to amplify single-stranded DNA for sequencing. Primers were designated and named by White *et al.* (1990). Primer sequences (5′ to 3′): "ITS2" = GCTGCGTTCTTCATCGATGC; "ITS3" = GCATCGATGAAGAACGCAGC; "ITS4" = TCCTCCGCTTATTGATATGC; "ITS5" = GGAAGTAAAAGTCGTAACAAGG.

merase (5 units/μl), 1.25 μl of 10 μM "ITS4" or "ITS2" primer, 0.63 μl of 1 μM "ITS5" or "ITS3" (with "ITS4" only) primer, and 12.5 μl of genomic DNA (1–10 ng). Reaction mixtures were sealed with a drop of mineral oil to prevent evaporation during thermal cycling. The first reaction cycle consisted of 1 min at 97°C to denature double-stranded template DNA, 1 min at 48°C to anneal primers to single-stranded template DNA, and 45 s at 72°C to extend primers. Primer extension time was gradually increased in the next 39 cycles by the addition of 4 s to each remaining 72°C period. To allow completion of unfinished DNA strands, a 7-min 72°C extension time followed completion of the 40 thermal cycles. Reactions were monitored by inclusion of positive and negative controls in each set of samples.

Single-stranded DNA PCR products were resolved by electrophoresis on 3% agarose mini-gels (2% FMC NuSieve: 1% FMC SeaKem) from ≤5 μl of each reaction mix, using 1× TAE as the gel buffer. Single-stranded products from amplification of the entire ITS region (using primers "ITS4" and "ITS5"; Fig. 1) migrated below the somewhat bolder double-stranded band of the same length (ca. 650 bp) to a position corresponding to a double-stranded DNA band of ca. 450 bp. Single-stranded bands from amplifications of the 5.8S and ITS 2 regions (using primers "ITS4" and "ITS3"; Fig. 1) migrated below the corresponding double-stranded products (ca. 400 bp) to the position of a double-stranded DNA band of ca. 300–350 bp. DNA amplifications using primers "ITS5" and "ITS2" together produced a double-stranded band of ca. 370 bp and a single-stranded band that migrated like a ca. 250- to 300-bp double-stranded fragment.

PCR-generated DNAs were purified by differential filtration in Millipore Ultrafree-MC tubes (Millipore UFC3 THK 00). Reaction products were diluted with 350 μl sterile water in the filter unit and spun in a microcentrifuge at a rotor speed sufficient to remove all but 40–50 μl of the reaction dilution in 5–10 min. This procedure was repeated four times to purify the single-stranded DNA sufficiently for sequencing. Purified DNA was recovered by inverting the filter units into 15-ml conical test tubes and spinning them at top speed in a tabletop centrifuge for 2 min.

The single-stranded DNAs were sequenced by the dideoxy method using the TAQuense kit (U.S. Biochemical), following the protocols detailed therein, with ^{35}S-dATP as the labeling agent. "ITS5" or "ITS3" (Fig. 1), the limiting primers in the PCR amplifications, were used as the sequencing primers in the corresponding reactions. 7-Deaza-dGTP was substituted for dGTP in these reactions to prevent base compressions in the 5.8S region. Up to 24 samples were electrophoresed on each 5% acrylamide gel by using each lane of two adjacent sharktooth combs with 2.9-mm point-to-point spacing. Gels were fixed in 5% methanol/5% glacial acetic acid for 30 min, transferred to 3MM Whatman paper, vacuum-dried at 80°C for 0.5–1 h, and exposed to autoradiographic film for at least 12 h.

Sequence Analysis

Boundaries of the coding and spacer regions were determined by comparison of the DNA sequences to those of *Daucus carota* and *Vicia faba* (Yokota *et al.*, 1989). In these two species the limits of the mature 18S, 5.8S, and 26S rRNAs have been defined by S1 nuclease mapping (Yokota *et al.*, 1989). DNA sequences were aligned manually by sequential pairwise comparisons (cf. Swofford and Olsen, 1990). Divergence at nucleotide positions between pairs of sequences was calculated from all unambiguously aligned positions where no gaps, polymorphisms, or nucleotide-state ambiguities were present.

The data matrix consisted of ITS sequences from four Hawaiian silversword alliance species in *Argyroxiphium, Dubautia,* and *Wilkesia* and seven Californian tarweed species in *Adenothamnus, Madia, Raillardella,* and *Raillardiopsis* (Table 1). These species were chosen because all but two were subjects of an earlier phylogenetic study using cpDNA restriction site variation (Baldwin, 1989; Baldwin *et al.*, 1991) with which the results from the present study could be compared.

ITS sequences from two Compositae species outside Madiinae, *Arnica mollis* and *Hulsea algida,* served as outgroups.

Nucleotide sites from ITS 1, 5.8S, and ITS 2 sequences with potential phylogenetic information, i.e., with at least two nucleotide states each present in at least two sequences, were included in a data matrix. The two *Madia bolanderi* sequences were merged in these analyses because they were identical at all potentially informative sites. Only those nucleotide sites with unambiguous alignments were used in the phylogenetic analysis. Gaps were treated as missing data. The resulting matrix was analyzed by Wagner parsimony using the PAUP (version 3.0R, D. L. Swofford, Illinois Natural History Survey) "branch-and-bound" option, with collapse of zero-length branches, to find the maximally parsimonious trees. Trees longer than the maximally parsimonious solutions were examined to determine the number of additional evolutionary steps required to collapse sister-group relationships in at least one of the shortest trees, i.e., to determine the decay index (cf. Donoghue *et al.,* 1992). Bootstrap values for particular clades were calculated from 100 replicate Wagner parsimony analyses using the PAUP "heuristics" option and "closest" addition sequence of the taxa. A tree-length distribution of 10,000 random parsimony trees was generated from the data matrix using the PAUP "random trees" selection. This distribution was analyzed for skewness as an estimate of phylogenetic signal in the ITS sequences (cf. Huelsenbeck, 1991).

A subset of data from an earlier phylogenetic study of Madiinae using cpDNA restriction site variation (Baldwin, 1989; Baldwin *et al.,* 1991) was reexamined by the same phylogenetic methodology described above to provide a parallel comparison with the ITS results. Only those accessions included in the ITS analyses were reanalyzed for cpDNA relationships. In addition, the cpDNA and ITS data sets were combined and analyzed together (cf. Hillis, 1987) to further explore their congruence and complementarity. Combined analyses necessitated deletion of ITS data from representatives of two species for which cpDNA information was not available: *Madia elegans* and *M. stebbinsii.*

RESULTS

ITS Structure, Size, and Composition

Organization of a typical eukaryotic nrDNA tandem repeat unit is shown in Fig. 1. In the Compositae species examined, ITS 1, the spacer flanked by the 18S and 5.8S subunits, was consistently larger than ITS2, the spacer flanked by the 5.8S and 26S subunits.

Length of the entire ITS region varied among the Madiinae DNAs from 638 to 647 bp. ITS 1 ranged in length from 255 to 261 bp. ITS 2 varied from 216 to 223 bp. Percentage G + C content spanned from 47.7 to 51.4% in ITS 1 and from 49.5 to 53.0% in ITS 2. The 5.8S subunit was uniform in size (164 bp) but was somewhat variable in percentage G + C content (51.2 to 53.7%). Aligned DNA sequences of ITS 1, the 5.8S subunit, and ITS 2 from the study species are presented in Table 2.

ITS Length Mutations

Alignment of ITS 1 sequences of all Madiinae DNAs required one or more gaps at 5.1% of sites. Addition of the two outgroup species required gaps at 8.1% of ITS 1 sites. Alignment of all Madiinae ITS 2 sequences necessitated one or more gaps at 6.3% of nucleotide positions. Inclusion of ITS 2 sequences of the outgroup species required gaps at 6.7% of sites. No gaps were needed to align all 5.8S sequences.

nrDNA Repeat-Unit Variants

No ITS length variants or major sequence variants were found in any of the DNAs examined. Fine-scale separation of PCR products on 3% agarose gels resolved only a single double-stranded band and one single-stranded band in every instance. In addition, no superimposition of major sequence variants was evident from autoradiographs; i.e., within individual sequences a minor proportion of sites were polymorphic for two or more nucleotide states (see Table 2).

Divergence between ITS Sequences

ITS 1. Within Madiinae, ITS 1 sequence divergence between pairs of species ranged from 0.4 to 19.2% of nucleotides (Table 3A). Among Californian Madiinae taxa, *Raillardiopsis scabrida* showed the least divergence from each of the Hawaiian species in ITS 1 sequence comparisons (7.9 to 8.7% of nucleotides). ITS 1 sequence divergence values from comparisons between Madiinae and outgroup species (10.8 to 22.9% of nucleotides) overlapped strongly with those from comparisons within Madiinae. For example, the congeners *Madia bolanderi* and *M. elegans* were more divergent from one another in ITS 1 sequences (16.7–17.1% of nucleotides) than were *Adenothamnus validus* and the outgroup species *Arnica mollis* (10.8% of nucleotides).

ITS 2. ITS 2 sequence divergence values from pairwise comparisons of Madiinae DNAs (0 to 12.9% of nucleotides; Table 3B) were mostly lower than values from corresponding ITS 1 sequence pairs. Among the Californian species, *Raillardiopsis muirii* was the least divergent in ITS 2 sequences from each of the Hawaiian taxa (3.1 to 6.2% of nucleotides). ITS 2 sequence divergence values from comparisons between Madiinae and outgroup species (7.2 to 19.6% of nucleotides) were all lower than values from corresponding ITS 1 comparisons. As with ITS 1, considerable overlap in ITS 2 sequence divergence values occurred between

TABLE 2

Aligned DNA Sequences of the Internal Transcribed Spacer Region (See Fig. 1) from 12 Madiinae and Two Outgroup Populations (See Table 1)

ITS 1 →

```
                  1         2         3         4         5         6
                  0         0         0         0         0         0
1*TCGAATCCTGCATAGCAGAACGACCCGTGAACACGTACAA-CAACATGGCCTAAAGAGGA
2*TCGAATCCTGCATAGCAGAACGACCCGTGAACATGTACAAACAACATGGCCTTACGGGGA
3 TCGAATCCTGCACAGCAGAACGACCCGTGAACACGTACAA-CAACATGGCCTCATGAGGA
4 TCGAATCCTGCATAGCAGAACGACYCGTGAACATGTACAA-CAACATGGCCTCATGAGGA
5 TCGAATCCTGCATAGCAGAACGACCCGTGAACATGTACAA-CAACATGGCCTCATGAGGA
6 TCGAATCCTGCATAGCAGAACGACCTGTGAACACGTAAAA-CAACATGGCCTTATAAGGA
7 TCGAATCCTTCACAGCAGAACGACCCGTGAACACGTACAA-CAACATGGCCTYATGAGGA
8 TCGAATCCTGCATAGCAGAACGACCCGTGAACACGTACAA-CAACATGGACTCATGGGGA
9 TCGAATCCTGCGTAGCAGAACGACCTGTGAACACGTAAAA-CAACATGGCCTCACGAGGA
10 TCGAATCCTGCACAGCAGAATGACCCGTGAACATGTACAA-CAACCTGGCCTCATGAGGA
11 TCGAATCCTGCACAGCAGAACGACCCGTGAACACGTACAA-CAACCTGGCCTCATGAGGA
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5.8S →

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                  2         2         2         2         2         3
                  5         6         7         8         9         0
                  0         0         0         0         0         0
1*GTGCT-TATTGTGCGTGGCTTCTTTCAAATCTTAAACGACTCTCGGCAACGGATATCTCG
2*-TGCG-CATTGCACCTGGCTTCTTTGTAATCTTAAACGACTCTCGGCAACGGATATCTCG
3 -TGTT-YATTGCCCGTGGCTTCTTTATAATCTTAAACGACTCTCGGCAACGGATATCTCG
4 -TGTT-CATTGCCCGTGGCTTCTTTCTAATCATAAACGACTCTCGGCAACGGATATCTCG
5 -TGTTTCATTGTTCGTGGCTTCTTTATAATCATAAAYGACTCTCGGCAACGGATATCTCG
6 -TGTT-CACTGTTCGTGGCCTCTTTGTAATCATAAACGACTCTCGGCAACGGATATCTCG
7 -TGTT-TACTGCATGTGGCTTCTTTACAATCATAAACGACTCTCGGCAACGGATATCTCG
8 -TGTT-CATTGTTTGTGGCTTCTTTACAATCATAAACGACTCTCGGCAACGGATATCTCG
9 -TGCT-CATTGTTCGTGGCTCCTTTATAATCATAAACGACTCTCGGCAACGGATATCTCG
10 -TGTT-CATTGTTCGTGGCTTCTTTTTAATCATAAACGACTCTCGGCAACGGATATCTCG
11 -TGTT-CATTGTTCGTGGCTTCTTTTTAATCATAAATGACTCTCGGCAACGGATATCTCG
12 -TGTT-CATTGTTCGTTGCTTCTTTATAATCATAAACGACTCTCGGCAACGGATATCTCG
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TABLE 2—*Continued*

```
          4         5         5         5         5         5
          9         0         1         2         3         4
          0         0         0         0         0         0

 1*TGTGGGCGGAGATTGGTCTCCCGTGGTAATGACGCGGTTGGCCTAAATATGAGTCCCATC
 2*TGTGGGCGGAGATTGGTCTCCTGTGCCCATGGCGCGGCTGGCCTAAATATGAGTCCCTTC
 3 TGTGGGCGGAGATTGGTCTCCCGTGTTCATGACGCGGTTGGCCTAAATACGAGTCCCATT
 4 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCCCATC
 5 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCCCGTC
 6 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCTTGTC
 7 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAAAGCGAGTCCTGTC
 8 TGTGGGCGGAGATTGGTCTCCCGTATCCATGATGTGGTTGGCCTAAATTTGAGTCTCGTT
 9 TGTGGGCGGATATTGGTCTCCCGTTTCCATGATGCGGTTGGCCTAAATAYGAGTCTCGTC
10 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCTCGTC
11 TGTGGGCGGAGATTGGTCTCCCGTGTCCAGTTTGCGGTTGGCTTAAATACGAGTCTTGTC
12 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCTTGTC
13 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAATACGAGTCTTGTC
14 TGTGGGCGGAGATTGGTCTCCCGTGTCCATGATGCGGTTGGCCTAAAAGCGAGTCCTGTC

          5         5         5         5         5         6
          5         6         7         8         9         0
          0         0         0         0         0         0

 1*AAGAGGGACGCACGACTARTGGTGGTTGATTACACAGTCGTCTCGTGTCGTGCGTCTTGA
 2*TAGAGGGATGCACGRCTAGTGGTGGTTGATTAAACAGTCGTCTCGTGTCGTGTGTTTTGA
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ITS 1 and ITS 2 combined. Comparison of Madiinae sequence pairs across both spacers gave divergence values ranging from 0.2 to 15.0% of nucleotides (Table 3C). Among the California tarweeds, *Raillardiopsis scabrida* showed the least combined ITS sequence divergence from each of the Hawaiian species (6.9 to 8.5% of nucleotides). Intersubtribal divergence values spanned from 9.2 to 20.5% of nucleotides.

5.8S nrDNA. Pairwise divergence values from comparisons of 5.8S sequences were much lower (0 to 2.5% of nucleotides; Table 3D) than those for either ITS region in corresponding DNAs.

ITS Nucleotide Site Variation

Alignment of all ITS region sequence positions resulted in a matrix of 659 characters (Table 2). Of these, it was deemed necessary to delete 9 positions from ITS 1 (sites 67–75; Table 2) and 17 positions from ITS 2

TABLE 3

Pairwise Divergence between ITS Region Sequences from 12 Madiinae and Two Outgroup DNAs (See Table 1)

A. ITS 1 distance matrix

	1	2	3	4	5	6	7	8
1	-	0.146	0.108	0.142	0.142	0.142	0.162	0.158
2	35	-	0.175	0.192	0.200	0.213	0.217	0.213
3	26	42	-	0.075	0.096	0.146	0.096	0.138
4	34	46	18	-	0.112	0.154	0.129	0.154
5	34	48	23	27	-	0.104	0.104	0.100
6	34	51	35	37	25	-	0.146	0.121
7	39	52	23	31	25	35	-	0.142
8	38	51	33	37	24	29	34	-
9	37	54	39	46	30	23	41	36
10	38	51	28	29	19	32	35	30
11	39	53	29	32	21	32	35	30
12	40	55	29	34	21	32	34	28
13	39	54	28	33	20	32	33	29
14	38	51	22	30	24	34	1	33

	9	10	11	12	13	14
1	0.154	0.158	0.162	0.167	0.162	0.158
2	0.225	0.213	0.221	0.229	0.225	0.213
3	0.162	0.117	0.121	0.121	0.117	0.092
4	0.192	0.121	0.133	0.142	0.138	0.125
5	0.125	0.079	0.087	0.087	0.083	0.100
6	0.096	0.133	0.133	0.133	0.133	0.142
7	0.171	0.146	0.146	0.142	0.138	0.004
8	0.150	0.125	0.125	0.117	0.121	0.138
9	-	0.158	0.158	0.158	0.154	0.167
10	38	-	0.025	0.033	0.029	0.142
11	38	6	-	0.033	0.029	0.142
12	38	8	8	-	0.004	0.138
13	37	7	7	1	-	0.133
14	40	34	34	33	32	-

B. ITS 2 distance matrix

	1	2	3	4	5	6	7	8
1	-	0.124	0.072	0.093	0.108	0.088	0.108	0.149
2	24	-	0.113	0.134	0.134	0.134	0.149	0.196
3	14	22	-	0.052	0.062	0.072	0.093	0.119
4	18	26	10	-	0.082	0.072	0.088	0.129
5	21	26	12	16	-	0.041	0.072	0.103
6	17	26	14	14	8	-	0.062	0.082
7	21	29	18	17	14	12	-	0.108
8	29	38	23	25	20	16	21	-
9	20	29	19	19	15	9	19	18
10	23	28	16	18	11	10	16	21
11	26	29	20	22	16	12	17	25
12	23	28	16	18	10	6	14	20
13	24	29	17	19	11	7	15	21
14	21	29	18	17	14	12	0	21

	9	10	11	12	13	14
1	0.103	0.119	0.134	0.119	0.124	0.108
2	0.149	0.144	0.149	0.144	0.149	0.149
3	0.098	0.082	0.103	0.082	0.088	0.093
4	0.098	0.093	0.113	0.093	0.098	0.088
5	0.077	0.057	0.082	0.052	0.057	0.072
6	0.046	0.052	0.062	0.031	0.036	0.062
7	0.098	0.082	0.088	0.072	0.077	0.000
8	0.093	0.108	0.129	0.103	0.108	0.108
9	-	0.077	0.098	0.077	0.082	0.098
10	15	-	0.041	0.021	0.026	0.082
11	19	8	-	0.031	0.036	0.088
12	15	4	6	-	0.005	0.072
13	16	5	7	1	-	0.077
14	19	16	17	14	15	-

C. Combined ITS 1 and ITS 2 distance matrix

	1	2	3	4	5	6	7	8
1	-	0.136	0.092	0.120	0.127	0.118	0.138	0.154
2	59	-	0.147	0.166	0.171	0.177	0.187	0.205
3	40	64	-	0.065	0.081	0.113	0.094	0.129
4	52	72	28	-	0.099	0.118	0.111	0.143
5	55	74	35	43	-	0.076	0.090	0.101
6	51	77	49	51	33	-	0.108	0.104
7	60	81	41	48	39	47	-	0.127
8	67	89	56	62	44	45	55	-
9	57	83	58	65	45	32	60	54
10	61	79	44	47	30	42	51	51
11	65	82	49	54	37	44	52	55
12	63	83	45	52	31	38	48	48
13	63	83	45	52	31	39	48	50
14	59	80	40	47	38	46	1	54

	9	10	11	12	13	14
1	0.131	0.141	0.150	0.145	0.145	0.136
2	0.191	0.182	0.189	0.191	0.191	0.184
3	0.134	0.101	0.113	0.104	0.104	0.092
4	0.150	0.108	0.124	0.120	0.120	0.108
5	0.104	0.069	0.085	0.071	0.071	0.088
6	0.074	0.097	0.101	0.088	0.090	0.106
7	0.138	0.118	0.120	0.111	0.111	0.002
8	0.124	0.118	0.127	0.111	0.115	0.124
9	-	0.122	0.131	0.122	0.122	0.136
10	53	-	0.032	0.028	0.028	0.115
11	57	14	-	0.032	0.032	0.118
12	53	12	14	-	0.005	0.108
13	53	12	14	2	-	0.108
14	59	50	51	47	47	-

D. 5.8S distance matrix

	1	2	3	4	5	6	7	8
1	-	0.019	0.019	0.006	0.006	0.006	0.012	0.006
2	3	-	0.025	0.012	0.012	0.012	0.006	0.012
3	3	4	-	0.012	0.012	0.012	0.019	0.012
4	1	2	2	-	0.000	0.000	0.006	0.000
5	1	2	2	0	-	0.000	0.006	0.000
6	1	2	2	0	0	-	0.006	0.000
7	2	1	3	1	1	1	-	0.006
8	1	2	2	0	0	0	1	-
9	1	2	2	0	0	0	1	0
10	3	4	4	2	2	2	3	2
11	1	2	2	0	0	0	1	0
12	2	3	3	1	1	1	2	1
13	2	3	3	1	1	1	2	1
14	2	1	3	1	1	1	0	1

	9	10	11	12	13	14
1	0.006	0.019	0.006	0.012	0.012	0.012
2	0.012	0.025	0.012	0.019	0.019	0.006
3	0.012	0.025	0.012	0.019	0.019	0.019
4	0.000	0.012	0.000	0.006	0.006	0.006
5	0.000	0.012	0.000	0.006	0.006	0.006
6	0.000	0.012	0.000	0.006	0.006	0.006
7	0.006	0.019	0.006	0.012	0.012	0.000
8	0.000	0.012	0.000	0.006	0.006	0.006
9	-	0.012	0.000	0.006	0.006	0.006
10	2	-	0.012	0.019	0.019	0.019
11	0	2	-	0.006	0.006	0.006
12	1	3	1	-	0.000	0.012
13	1	3	1	0	-	0.012
14	1	3	1	2	2	-

Note. Divergence values in the upper right half of each matrix are the proportion of divergent sites in each comparison. Actual numbers of divergent sites appear in the lower left half of each matrix. Sequences were compared only at unambiguously aligned positions that lacked ambiguous, missing, or polymorphic states. Numbers along the top and left margins of each matrix correspond to sample numbers in Table 2.

parsimonious trees. Bootstrap values for clades that were resolved similarly in all trees ranged from 55 to 100%. Among the alternative topologies, tree's E and F (in Fig. 2) received greatest support from the bootstrap analysis. Separate Wagner branch-and-bound analyses of ITS 1 data alone resulted in three shortest trees. The strict consensus of these trees was compatible with, but slightly less resolved than, the strict consensus tree from analysis of the combined ITS data set (Fig. 3). Similar analysis of the much smaller ITS 2 data set yielded one maximally parsimonious tree that was compatible with the strict consensus from the com-

TABLE 4

Data Matrix of Potentially Phylogenetically Informative Nucleotide Positions from the ITS Region of 11 Madiinae and Two Outgroup DNAs (See Table 1)

```
                    1111111111111111111111222222222222222224
  1233455566788889999900011111222233444455790011122444555566 70
  3648635723813450123803523589067824568908942915848479234767 28

 1*TCCCAAAACGTCGTCTTAACTATTTAATCTTTGCCAGCTCTCACYCAACCTTTGCGCATT
 2*TCTCATCGGTTCTTCTTAGCCATGCAGTCTTTGCCAGTTCTAACTTAGCCCTCACGGTTC
  3 CCCCACTACCTCGGCTGGACTACTAAATCTGTGCCGGTTCCTATCCGAATYTCCCGATTT
  4 TCTCACTATCTCAGCTGGATTGCTAAGTCTTTGCGGGTCCCCATTCGAATCTCCCGCTAT
  5 TCTCACTACTCCGTCTGTGCTGCTTGATTTGCTCGGGTTCCCATCCGTATCTTTCGATAT
  6 TTCAATTATGACCCCTGTGCTGTTTGATTTGCGCGACCTTTCACTCTTATCCTTCGGTAT
  7 CCCCAYTACTTCGGCTGTGCTACTGAATTTCCAGGGGTTTCCATTCGAATTCCATGACAC
  8 TCCCACTGCTATCTCTGTGCCGTTTGATATGCGCAATTCCCCATCCTAATCTTTTGACAT
  9 TTCAACCACGATGCCTGTGCTGTTTGATCTGCTGGAGCTTTAACCCTTACCTTTCGATAT
 10 CCTCCCTACTCCGTCCGTGTTGCCTGAGCGGTGCGTGTCCCCTTCCTTATCTTTCGTTAT
 11 CCCCCCTACTCCGTCCGTCTTGCCTGAGCGGCGCGTGTCCCCTTCTTTATCTTTCGTTAT
 12 CCCCCCTACTCCGTTCGTGTTTCCTGAGCGGCGCGTTTCCCCTTCCTTATCTTTCTATAT
 13 CCCCCCTACTCCGTTCGTGTTTCCTGAGCGGCGCGTGTCCCCTTCCTTATCTTTCTATAT

    4444555555555555555556666666666666
    1347013333444489999990011113334 55
    0414730678015993467891801261345 17

 1*GCCCTCTCCACAGCCCGCTTGTGRGTGTATCGC
 2*GCYTCCTCCTCTGTCTGTTTGTGGAAATATGAC
  3 RCCCTCCCCATGTTCCGTTTGTAGGAACACCAY
  4 GCCCCTCCCACGTCTCGTTYGTAGGAACATCAC
  5 GCCTCTCCCGCGTTCCGTTTGTACGGACGCTGC
  6 GCTTCTCTTGCGTCCCGCTTGTATGGACGTTGC
  7 GCTTCTCCTGCATCTCGTTTGTATGGATACTGC
  8 GCCTCTTTCGTGTCTCGCTCGCACGGAGTCTGC
  9 GCCTCTYTCGCGTCCTGCTTGCATGGGCATTGC
 10 GCCTCTCTCGCGTCCCCTCCTTACAGACACTGT
 11 GCCTCTCTTGCTTCCCCTCCTTACAGACACTGT
 12 ATCTCTCTTGCGTCCCCTCCTTACAGACGCTGT
 13 ATCTCTCTTGCGTCCCCTCCTTACAGACGCTGT
```

Note. Vertical columns are nucleotide positions as given in Table 2. Horizontal rows are nucleotide states from individual sequences. Numbers along left margin correspond to sample numbers in Table 2. Asterisks denote outgroup sequences. Sequence symbols: A, C, G, T = dATP, dCTP, dGTP, dTTP; Y = C or T; hyphens = gaps or missing nucleotides; question marks = nucleotides of unknown identity. Sequence 14 was deleted from the matrix because it is identical to sequence 7 at all potentially informative sites.

bined ITS data (Fig. 3) except for placement of the *Madia elegans/Raillardiopsis muirii* clade as the sister group to the lineage including the silversword alliance and the remaining *Madia* and *Raillardiopsis* species.

Reanalysis of the cpDNA restriction site data from the same Hawaiian and Californian species accessions (minus *Madia elegans* and *M. stebbinsii*) resulted in seven maximally parsimonious trees of 74 steps, excluding uninformative variation. The strict consensus of these seven trees is given in Fig. 4. The consistency index of each of the seven trees was 0.85, including only potentially informative sites. Their retention index was 0.91.

Analysis of the combined ITS and cpDNA data matrix yielded two fully resolved maximally parsimonious trees (consistency index = 0.71; retention index = 0.74) that differed only in their resolution of the relationship among *Adenothamnus, Raillardella,* and the *Madia/Raillardiopsis*/silversword alliance lineage. Neither of these two trees was identical to any of the topologies obtained from separate analysis of the cpDNA or ITS data, although each combined-data clade also appeared in at least two of the shortest cpDNA or ITS trees. The strict consensus of both combined-data trees is presented in Fig. 5.

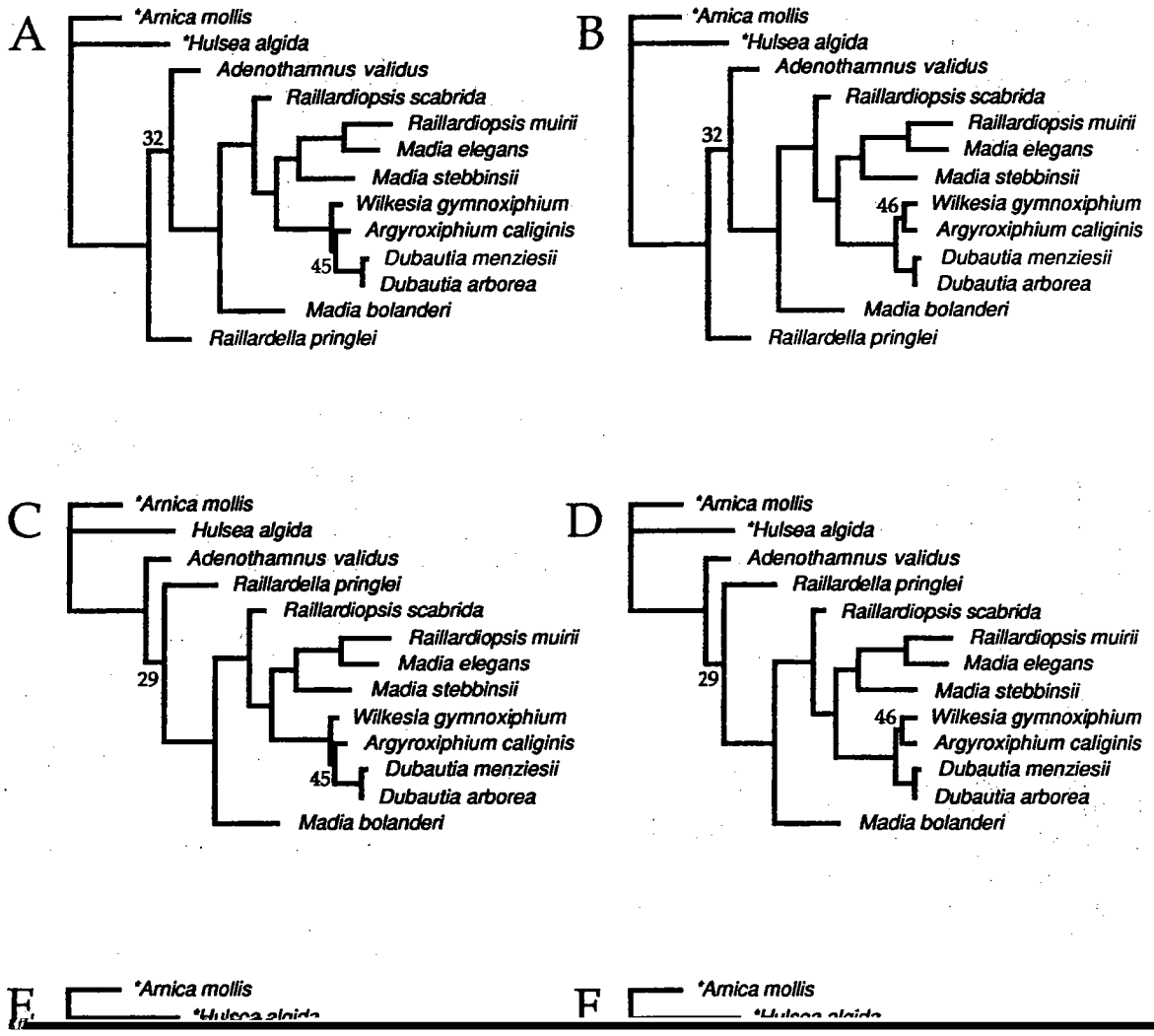
DISCUSSION

ITS Sequence Comparisons

Organization of the ITS region in the Compositae species investigated is similar to that found in other angiosperms (Rogers and Bendich, 1987) and higher eukaryotes in general (Appels and Honeycutt, 1986). ITS 1 and ITS 2 are only slightly variable in size among the few flowering plants for which data have been reported (*Oryza sativa,* Takaiwa *et al.,* 1985; *Cucumis melo,* Kavanagh and Timmis, 1988; *Lycopersicon esculentum,* Kiss *et al.,* 1988; *Sinapis alba,* Rathgeber and Capesius, 1989; *Vigna radiata,* Schiebel and Hemleben, 1989; *Daucus carota* and *Vicia faba,* Yokota *et al.,* 1989; *Nicotiana rustica,* Venkateswarlu and Nazar, 1991). In most of the previously reported angiosperm sequences, however, ITS 2 is larger than ITS 1. The Compositae species studied here are similar to *Vicia faba* and *Sinapis alba* in having an ITS 1 that is larger than ITS 2.

The narrow span in size of ITS 1 among the Compositae studied (255 to 261 bp) falls near the upper extreme of the ITS 1 size range reported from the above seven dicotyledons and one monocotyledon (194 to 265 bp). The size range of ITS 2 among the study species (216 to 223 bp) lies within the upper intermediate range of that reported from other angiosperms (188 to 237 bp). The size of the Compositae 5.8S subunit (164 bp) is the same as one of the two lengths of this region reported from other flowering plants (163–164 bp).

In contrast to the narrow range of ITS size variation in angiosperms apparent from sequence length comparisons, Jorgensen and Cluster (1988) reported ITS restriction fragment variants differing by as much as 200 bp in a study encompassing nine legume genera from seven tribes. In addition, ITS length variation has been reported within the single species *Lisianthius skinneri* (Gentianaceae). An individual representing one population was fixed for an ITS deletion of 50 bp (Sytsma and Schaal, 1985). Another population contained individuals with two apparent ITS 1 length variants that differed by ca. 100 bp (Sytsma and Schaal, 1990). Sytsma and Schaal (1990) speculated that this within-individual variation may be explained by multiple rDNA loci in this presumably tetraploid species. The lack of such length variants or significant sequence variants in the tetraploid Compositae species examined here (i.e., in the silversword alliance, *Adenothamnus,* or *Raillardella* DNAs) may indicate origin by autopolyploidy or amphiploidy involving species


A
*Arnica mollis
*Hulsea algida
Adenothamnus validus
Raillardiopsis scabrida
Raillardiopsis muirii
Madia elegans
Madia stebbinsii
Wilkesia gymnoxiphium
Argyroxiphium caliginis
Dubautia menziesii
Dubautia arborea
Madia bolanderi
Raillardella pringlei
32
45
B
*Arnica mollis
*Hulsea algida
Adenothamnus validus
Raillardiopsis scabrida
Raillardiopsis muirii
Madia elegans
Madia stebbinsii
Wilkesia gymnoxiphium
Argyroxiphium caliginis
Dubautia menziesii
Dubautia arborea
Madia bolanderi
Raillardella pringlei
32
46
C
*Arnica mollis
Hulsea algida
Adenothamnus validus
Raillardella pringlei
Raillardiopsis scabrida
Raillardiopsis muirii
Madia elegans
Madia stebbinsii
Wilkesia gymnoxiphium
Argyroxiphium caliginis
Dubautia menziesii
Dubautia arborea
Madia bolanderi
29
45
D
*Arnica mollis
*Hulsea algida
Adenothamnus validus
Raillardella pringlei
Raillardiopsis scabrida
Raillardiopsis muirii
Madia elegans
Madia stebbinsii
Wilkesia gymnoxiphium
Argyroxiphium caliginis
Dubautia menziesii
Dubautia arborea
Madia bolanderi
29
46
E
*Arnica mollis
F
*Arnica mollis

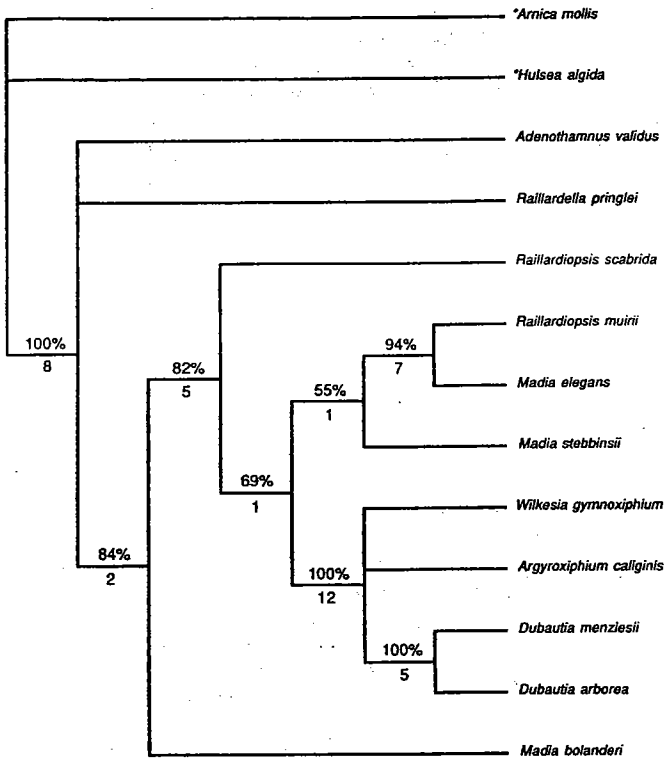


FIG. 3. Strict consensus of the six maximally parsimonious Wagner ITS trees in Fig. 2. Asterisks designate outgroup species (*A. mollis* and *H. algida*). Percentages above branches are bootstrap values. Numerals below branches indicate the number of additional evolutionary steps required to collapse the corresponding clade in at least one of the maximally parsimonious trees (i.e., the decay index, cf. Donoghue *et al.*, 1992).

Such sequence conservation among the ITS sequences of closely related species is not too surprising. Evidence from experimental and computer-simulation studies (Thweatt and Lee, 1990; Gonzalez *et al.*, 1990a; Venkateswarlu and Nazar, 1991) suggests that ITS sequences are under evolutionary constraint because of an important role in processing mature rRNAs from primary transcripts. Secondary, "crucifix or tRNA-like core" (Venkateswarlu and Nazar, 1991) structures assumed by both ITS units in the primary rRNA transcripts may be critical to rRNA maturation by bringing the ends of the 18S, 5.8S, and 26S rRNA regions into close proximity for processing.

Stabilizing selection on the ITS region has not, however, been sufficient to preclude broad-scale evolutionary diversity in ITS secondary structures. Gonzalez *et al.* (1990b) reported that unalignable ITS 2 sequences of mouse and human are radically different in secondary structure based on rRNA electron micrographs of Wellauer *et al.* (1974). Estimates of angiosperm ITS secondary structures have shown that the shapes of the cross-like configurations assumed by these sequences can vary widely among distantly related species (Venkateswarlu and Nazar, 1991). Poor alignability of the Compositae ITS sequences reported here with those of species from other plant families also suggests lack of long-term evolutionary constraint on much of the ITS region.

The wide range of variation in base composition already known among the few ITS sequences in angiosperms for which data are available (see above) also suggests diversity in the type and extent of secondary structures assumed by transcripts of these regions from species in different families. Percentage G + C content values for ITS 1 (47.7 to 51.4%) and ITS 2 (49.5 to 53.0%) sequences of the Madiinae species reported here are among the lowest and most equitable reported in angiosperms. In contrast, G + C content in *Oryza sativa* ITS 1 (72.2%) and ITS 2 (77.3%) (Takaiwa *et al.*, 1985), the only monocotyledon sequences available, are higher and more highly skewed than any reported dicotyledon ITS sequences.

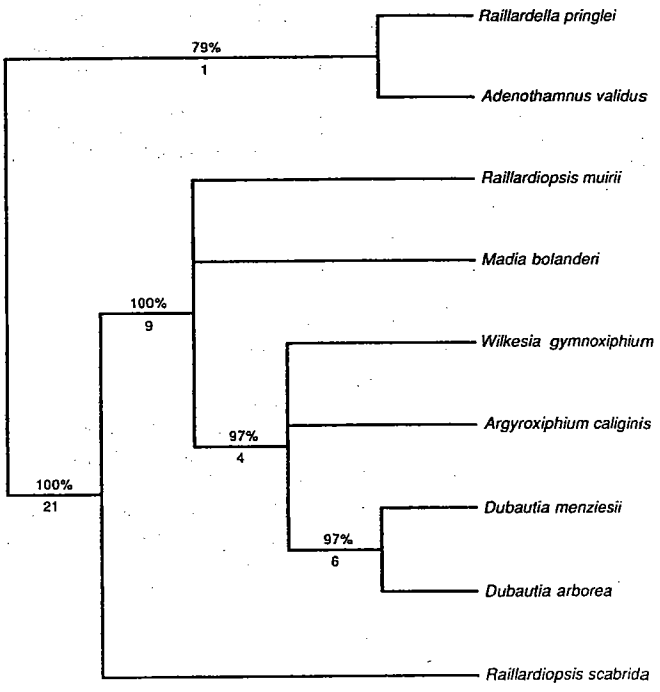


FIG. 4. Strict consensus of the seven maximally parsimonious Wagner trees from analysis of cpDNA restriction sites among the same Californian and Hawaiian species accessions examined for ITS sequence relationships (excluding *M. elegans* and *M. stebbinsii*, for which data were not available). Polarity was determined by outgroup comparison to four species: *Arnica mollis, Encelia densifolia, Helenium bigelovii,* and *Hulsea algida*. Percentages above branches are bootstrap values from 100 replicate branch-and-bound Wagner analyses. Numerals below branches indicate the number of additional steps required to collapse the corresponding clade in at least one of the maximally parsimonious trees (i.e., the decay index, cf. Donoghue *et al.*, 1992). Consistency index = 0.85. Retention index = 0.91. This analysis is a subset of that of Baldwin (1989) and Baldwin *et al.* (1991).

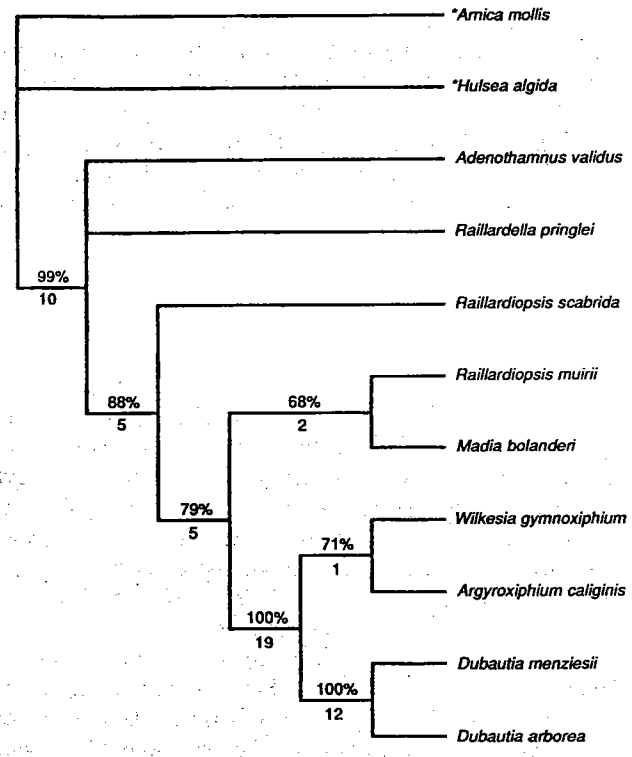


FIG. 5. Strict consensus of the two maximally parsimonious Wagner trees from analysis of combined ITS sequence variation and chloroplast DNA restriction site variation among species of the Hawaiian silversword alliance species in *Argyroxiphium, Dubautia,* and *Wilkesia* and Californian tarweeds in *Adenothamnus, Madia, Raillardella,* and *Raillardiopsis.* Asterisks denote outgroup species (*A. mollis* and *H. algida*). Percentages above branches are bootstrap values. Numerals below branches indicate the number of additional evolutionary steps required to collapse the corresponding clade in at least one of the maximally parsimonious trees (i.e., the decay index, cf. Donoghue *et al.*, 1992).

ITS Phylogenetic Resolutions

Extensive divergence of ITS sequences (>10%) in several Madiinae species pairs raised concerns about the utility of the ITS region for assessing phylogeny of these taxa, i.e., that homoplasy would outweigh phylogenetic signal in the Wagner parsimony analyses. Inclusion of extrasubtribal outgroup species (*Amica* homoplasy in the ITS data was also indicated by conflicting results between Wagner parsimony trees based on either ITS 1 or ITS 2 sequences alone. Despite these indications of high parallelism in the ITS data, topological comparison of the ITS trees, cpDNA trees, and combined ITS and cpDNA trees suggests that phylogenetic signal prevailed in the phylogenies that were based on the entire ITS region. In addition, a g_1 value of -0.8 from analysis of the random tree distribution indicates phylogenetic signal in the data set (cf. Huelsenbeck, 1991).

The strict consensus of the six ITS trees (Fig. 3) is completely compatible topologically with the cpDNA strict consensus tree (Fig. 4) except for the alternative placements of *Madia bolanderi.* The cpDNA and ITS trees suggest that (a) the silversword alliance (*Argyroxiphium, Dubautia, Wilkesia*) is a monophyletic group descended from California tarweeds, (b) the Hawaiian group is more closely related to *Madia* and *Raillardiopsis* than to *Adenothamnus* or *Raillardella,* and (c) *Raillardiopsis,* a segregate of *Raillardella,* is a paraphyletic genus whose members show closer relationship to *Madia* and the silversword alliance than to *Raillardella.* In fact, both sets of trees suggest that the silversword alliance emerged from within *Raillardiopsis.* Complementarity of the ITS and cpDNA data is especially pronounced in the resolution of monophyly of the silversword alliance species, as best seen in the combined-data consensus tree (Fig. 5). In addition, the ITS trees strongly indicate monophyly of the Madiinae species relative to the two extrasubtribal outgroup species.

The ITS trees (Figs. 2 and 3) corroborate two unexpected findings from the cpDNA phylogeny (Fig. 4). First, both data sets indicate that *Madia, Raillardiopsis,* and the Hawaiian silversword alliance form a lineage exclusive of *Adenothamnus* and *Raillardella.* The monotypic *A. validus* had seemed an excellent candidate for closest-extant relative of the Hawaiian species, based on shared base chromosome number (n = 14), reproductive characters, and shrubby habit (Carr *et al.*, 1989). Second, despite Rydberg's (1927) segregation of *R. muirii* and *R. scabrida* from *Raillardella*

pringlei to each other and to the species of *Madia*, *Raillardiopsis*, and the silversword alliance. *Raillardella* and the Hawaiian species. As expected, all trees from the combined analysis of cpDNA and ITS data (Fig. 5)

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